An Approach to Trapping γ-Glutamyl Radical Intermediates Proposed for Vitamin K Dependent Carboxylase: α,β-Methyleneglutamic Acid[1]

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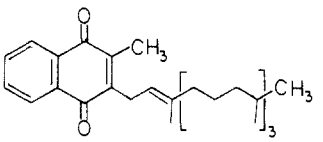
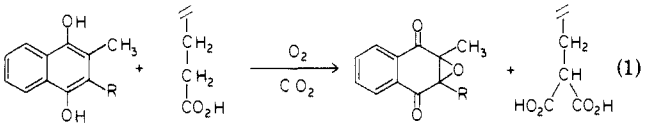
The vitamin K dependent carboxylase activates the glutamyl γ-CH of substrate peptides for carboxylation by producing a γ-glutamyl free radical, a γ-glutamyl carbanion, or through a concerted carboxylation. We propose to intercept the putative γ-glutamyl free radical by the intramolecular rearrangement of a substrate containing the α,β-cyclopropane analogue of glutamic acid. The rearrangement of cyclopropylcarbinyl radicals into 2-butenyl radicals is rapid, exothermic, and considered diagnostic of free-radical formation. 1-Amino-2-(carboxymethyl)cyclopropane-1-carboxylate, the β-cyclopropane analogue of glutamic acid, was synthesized starting from diethyl α-ketoglutarate. The α-keto ester was first treated with benzonitrile in sulfuric acid, to yield diethyl α,α-dibenzamidoglutarate. The α,α-dibenzamido acid was cleaved to produce the α,β-dehydroamino acid and benzamide on treatment with *p*-toluenesulfonic acid in hot benzene. Diazomethane addition to the dehydroamino acid resulted in cycloaddition of diazomethane and production of the pyrazoline, which upon irradiation lost N_2 to give the protected cyclopropane-containing amino acid analogue. Acidic hydrolysis of the *N*-benzoyl-α,β-methyleneglutamate diethyl ester resulted in the production of the unprotected amino acid, α,β-methyleneglutamic acid, in high yield. A single dehydroamino acid and a single methyleneglutamic acid isomer were produced in this synthesis; both are identified as the *Z* isomer, the former by NMR using the nuclear Overhauser effect and the latter through X-ray crystallographic analysis of *N*-benzoyl-α,β-methyleneglutamate diethyl ester. Saponification of a N-protected methyleneglutamic acid dialkyl ester using limiting alkali was shown to selectively yield the α-alkyl ester γ-acid. The reaction was used to produce α,β-cyclopropane-containing analogues of the carboxylase substrates *N*-*t*-Boc-L-glutamic acid α-benzyl ester and *N*-benzoyl-L-glutamic acid α-ethyl ester. The cyclpropane-containing analogues were tested and found to be neither substrates for nor inhibitors of the rat liver microsomal vitamin K dependent carboxylase. The inability of the enzyme to recognize these substrate analogues is attributed to the α-alkyl substitution, which apparently abolishes substrate binding.

Vitamin K or phylloquinone is a cofactor for a liver microsomal enzyme system which catalyzes the post-translational modification of precursors of prothrombin and other serum proteins.[2-4] The modification is the



Vitamin K_1: 2-methyl-3-phytyl-1,4-napthoquinone

carboxylation of specific protein-bound glutamyl side chains producing γ-carboxyglutamyl residues. The carboxylation reaction requires reduced vitamin K, oxygen, carbon dioxide, and protein-bound glutamyl residues as substrates. The products are γ-carboxyglutamyl residues, vitamin K epoxide, and water (eq 1). Current mechanisms



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for this unusual carboxylation propose that the role of the reduced vitamin is to activate the glutamyl γ-CH.[5-7] Such activation has been postulated to occur through abstraction of a hydrogen radical and concomitant production of a γ-glutamyl free radical,[8,9] by abstraction of one of the γ-glutamyl protons by a strong base and production of a carbanion,[6,7] or in a concerted carboxylation.[3,10] Both the existence of the γ-glutamyl intermediate and its identity are currently controversial, as each mechanism enjoys some experimental support. Furthermore, mechanisms invoking either the γ-glutamyl free radical or the γ-glutamyl carbanion are not mutually exclusive since the radical can generate the carbanion upon its one-electron reduction.

The ability to distinguish between the radical, the carbanionic, and the concerted carboxylations would advance the understanding of this mechanism. Attempts to trap a carbanion through the β-elimination of 3-fluoroglutamyl-containing substrates are already in progress.[11] We propose to intercept the γ-glutamyl free radical by using the intramolecular rearrangement of cyclopropylcarbinyl radicals into 3-butenyl radicals. This intramo-

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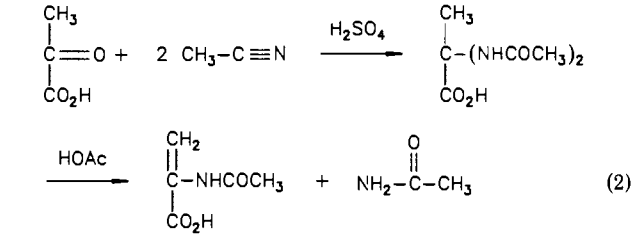
lecular rearrangement is rapid, exothermic, and sufficiently well documented that observation of the rearrangement is considered diagnostic of free-radical formation.[12-15] Application of this method to the vitamin K dependent carboxylase requires the production of a cyclopropane-substituted glutamic acid. We first considered the α,β-cyclopropane analogues of glutamic acid since these derivatives position the cyclopropane ring adjacent to the γ-carbon with the minimal structural change (i.e. addition of only a single carbon). Synthetic peptide[16] or amino acid[17] substrates for the carboxylase-containing methyleneglutamyl residues should be recognized and processed by the carboxylase. Radical intermediates, if formed, would be detected by the identification of the characteristic rearranged products. Since one fate of the rearranged radical is reaction with the enzymic active site, these cyclopropane-containing substrates might also be mechanism-based irreversible inhibitors of the carboxylase, and therefore candidates for anticoagulant drugs.

Results

Synthesis of the α,β-Cyclopropane Analogue of Glutamic Acid. α,β-Cyclopropane-containing amino acids have been synthesized by the addition of diazomethane to an unsaturated oxazolone,[18] by the addition of diazomethane to the α,β-dehydroamino acid followed by elimination of N_2 from the resulting pyrazoline,[19] and through the reaction of appropriately substituted and activated methylene compounds with a 1,2-haloalkane.[20] The reported availability of methyl esters of *N*-(benzyloxycarbonyl)-α,β-dehydroglutamate[21] motivated us to investigate the method utilizing diazomethane addition to the unsaturated amino acid. We were however, unable to successfully duplicate the condensation of dimethyl α-ketoglutaric acid with benzyl carbamate, which was reported[21] to give the unsaturated amino acid derivative γ-methyl-α-*N*-(benzyloxycarbonyl)-α,β-dehydroglutamate. We therefore sought alternate methods for the production of α,β-dehydroglutamic acid derivatives.

A successful method was developed on the basis of the observation that α,α-diacetamidopropionic acid is cleaved on treatment with glacial acetic acid at reflux, producing α-acetamidoacrylic acid (eq 2).[22,23] α,α-Diacetamido-propionic acids are in turn available by the slow addition of a nitrile to a mixture of pyruvic acid and cold concentrated sulfuric acid, followed by quenching of the reaction on ice (eq 2).[24]



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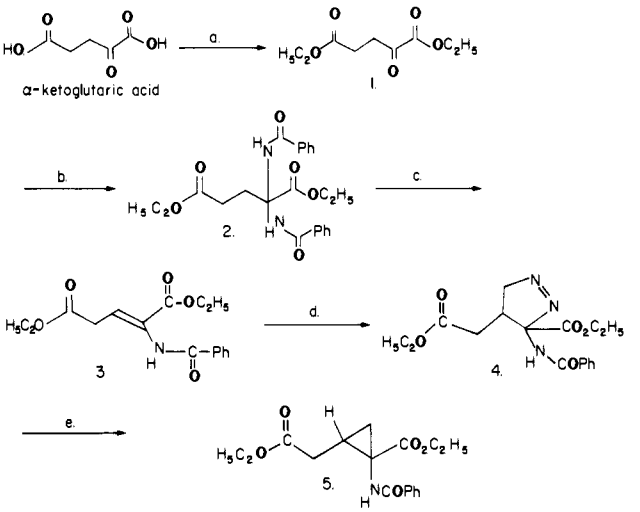
Scheme I. Synthesis of *N*-Benzoyl-α,β-methyleneglutamic Acid from α-Ketoglutaric Acid[a]



[a] (a) CH_3CH_2OH/HCl; (b) C_6H_6CN in concentrated H_2SO_4; (c) *p*-toluenesulfonic acid in benzene at reflux; (d) CH_2N_2 in ether for 18 h; (e) *hν*, 250 nm.

Our synthesis of *N*-benzoyl-α,β-methyleneglutamic acid diethyl ester (**5**) is depicted in Scheme I. Treatment of diethyl α-ketoglutarate (**1**) dissolved in concentrated sulfuric acid with benzonitrile followed by quenching of the reaction with ice–water and isolation of the precipitated product led to the production of α,α-dibenzamidoglutarate (**2**). Contrary to previous experience in the pyruvate series,[22] diethyl α-ketoglutarate (**1**) did not react with acetonitrile or benzyl cyanide to produce the corresponding α,α-diacylamino adduct. The attempted conversion of **2** to **3** by refluxing **2** in acetic acid was not successful. Various other acids and conditions for cleavage were evaluated, but either starting material was recovered unchanged or extensive decomposition resulted. Finally, we successfully developed a procedure for converting **2** into **3** by treatment of α,α-dibenzamidoglutarate with *p*-toluenesulfonic acid in refluxing benzene.

Olefin **3** was treated with diazomethane to produce 4-(carbethoxymethyl)-3-benzamido-3-carbethoxy-4-pyrazoline (**4**). Although this compound can be isolated, it is sufficiently unstable that its lengthy purification results in low isolated yields. Therefore pyrazoline **4** was

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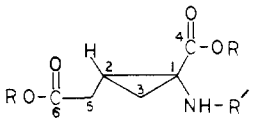
Table I. ^{1}H NMR Spectral Data of Methyleneglutamic Acid Derivatives



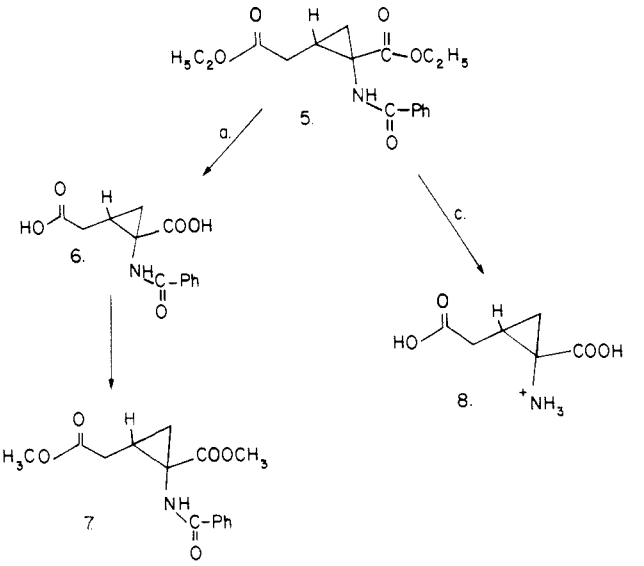
proton	chemical shift, ppm; multiplicity; and coupling constants, Hz		
	5 R = CH_2CH_3 R′ = COPh	7 R = CH_3 R′ = CoPh	8 R = R′ = H
H_2	2.03 (m)	1.91–2.05 (m)	2.12 (m)
H_{3A}	2.03 (m)	1.91–2.05 (m)	1.79 (dd) J = 8 and 10 Hz
H_{3B}	ca. 1.2	1.13 (dd) J = 4 and 6 Hz	1.31 (t) J = 7 Hz
H_{5A}	2.78 (dd) J = 5 and 16 Hz	2.72 (dd) J = 5 and 16 Hz	2.75 (dd) J = 8 and 18 Hz
H_{5B}	2.31 (dd) J = 10 and 16 Hz	2.26 (dd) J = 9 and 16 Hz	2.61 (dd) J = 8 and 18 Hz
R	4.16 and 4.21 (q) 1.22 and 1.30 (t) J = 7 Hz	3.63 and 3.69 (s)	
R′	7.43–7.54 (m, 3 H) 7.81–7.83 (m, 2 H)	7.3–7.5 (m, 3 H) 7.7–7.9 (m, 2 H)	

routinely used immediately and without further purification in the next step of the synthesis. Pyrolysis of pyrazoline 4 resulted in decomposition without production of cyclopropane 5. Irradiation of 4 with short-wavelength ultraviolet light (e.g. 250 nm) resulted in its clean and high-yield conversion to ethyl 2-(carbethoxymethyl)-1-benzamidocyclopropane-1-carboxylate (5). The yield and the purity of 5 were both highly dependent on the conditions of the irradiation, and temperature control as well as the presence of a sensitizer (e.g. benzene) were necessary to achieve best results.

The proton NMR of the fully protected methyleneglutamic acid 5 (0.002 M in $CDCl_3$; see Table I) initially caused us some concern, since the signals from two of the cyclopropane protons overlapped, producing a relatively sharp peak at 2.02 ppm, while the third cyclopropane signal could not be directly observed as it apparently overlapped with one of the methyl resonances. The chemical shifts and the splitting patterns were further found to be highly solvent as well as concentration dependent. In concentrated solutions (0.6 M, $CDCl_3$), the overlapping cyclopropyl peaks previously at 2.02 ppm were shifted upfield by 0.02 and 0.1 ppm and separated into two doublets of doublets. Despite these complications, a consistent set of proton assignments can be made (Table I) and supported using two-dimensional NMR techniques (COSY and C/H correlation).[25] Further, saponification of 5, followed by treatment of the resulting *N*-benzoyl diacid (6) with diazomethane, lead to production of dimethyl ester 7 (Scheme II). In the NMR spectrum of *N*-benzoyl dimethyl ester 7 the high-field cyclopropyl signal can be observed directly as a doublet of doublets at 1.20 ppm (Table I). The cyclopropane-containing structure assigned to 5 is also supported by the ^{13}C NMR (Table II), where three high-field cyclopropane resonances, two with characteristically high C–H coupling constants, are observed at 22.9, 23.9, and 37.2 ppm.

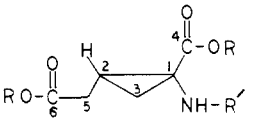
The fully deprotected amino acid α,β-methyleneglutamic acid (8) is available from 5 by acid hydrolysis (6 N HCl at reflux for 8 h).

Scheme II. Deprotection of 5[a]



[a] (a) LiOH in CH_3OH and H_2O, 0–5 °C; (b) CH_2N_2; (c) 6 N HCl, 75 °C.

Table II. ^{13}C NMR Spectral Data of Methyleneglutamic Acid Derivatives[a]



carbon no.	chemical shift,[b] ppm			
	5 R = CH_2CH_3 R′ = COPh	6 R = H R′ = COPh	7 R = CH_3 R′ = COPh	8 R = R′ = H
1	37.22	38.08	37.62	40.05
2	23.88	24.37	24.12	23.08
3	22.92	22.37	23.05	20.93
5	34.25	33.19	34.20	34.04
O ‖ –C–	171.83 172.80 168.62	176.55 177.36 173.13	172.18 173.26 168.66	174.31 177.11

[a] For the sake of clarity, chemical shifts for the alkyl and aromatic protecting groups of 5–7 have been omitted. [b] Measurement from TMS in $CDCl_3$ for 5–7; from TSP in D_2O for 8.

Stereochemistry. The configuration of olefin 3 and cyclopropylamino acid 5 remained to be established, since either the *Z* isomer, the *E* isomer, or a mixture of the two could result from this synthesis. Extensive chromatographic and spectroscopic examination of 3 as well as 5 have failed to detect inhomogeneity in the product. We conclude therefore that a single isomer of each has been formed. Our attempts to produce the other isomer of olefin 3 and thus deduce the relative stereochemistry by comparison of the properties of the isomeric olefins have not been successful. We therefore turned to the application of difference nuclear Overhauser (difference NOE) effects. The NOE effect has already been used for assignment of configurations to *E*- and *Z*-dehydrophenylalanine derivatives, where irradiation of the single olefinic signal produces an NOE-derived enhancement of the amide hydrogen for the *E*-dehydrophenylalanine.[26] The technique should be equally applicable to olefin 3. Irradiation of the two-proton

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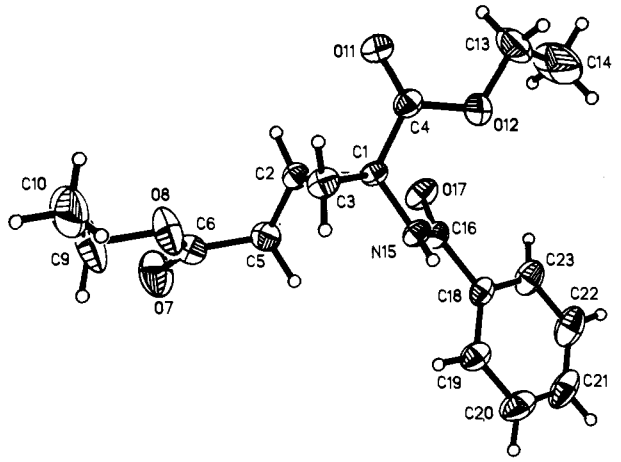


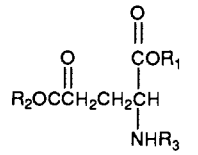
Figure 1.

methylene doublet at 3.35 ppm in **3** led to an increase in intensity of the amide signal at 7.90 ppm. Conversely, saturation of the olefinic one-proton triplet at 6.94 ppm produced no increase in amide intensity. Consequently, we conclude that the dehydroamino acid is the *Z* olefin as drawn for **3**.

We turned to X-ray crystallographic analysis in order to determine the stereochemistry of **5**. Crystallization from mixtures of carbon tetrachloride and hexanes yielded crystals suitable for X-ray analysis. A thermal ellipsoid plot of **5** is shown in Figure 1, which establishes it as the *Z* stereoisomer.

It is of interest to compare the structure of **5** to that of simple Glu-containing peptides. The incorporation of the cyclopropyl group at the Cα–Cβ site of a glutamic acid derivative imparts a degree of stereochemical rigidity into the molecule. The amide N (N15) and the γ C (C5) are syn with a torsion angle, χ^1, of −5.6(6)° about C1–C2, corresponding to an eclipsed conformation, as contrasted with the commonly observed values of close to 180° or to ±120° or ±60° for the energetically more favorable anti and gauche conformations, respectively. χ^2 for **5** (C1–C2–C5–C6) is nearly anti with a torsion angle of 156.1 (4)°. These values are in contrast to χ^1 and χ^2 of −44.5° and 71.1° and −56.6° and 78.8° for the glutamyl side chains of a fully blocked Glu-Glu peptide, α-*tert*-butyl-*N*-[*N*-(*tert*-butoxycarbonyl)-O^5-benzyl-α-L-glutamyl]-O^5-L-glutamate,[27] corresponding to a gauche–anti and a gauche–gauche conformation for the side chains. A gauche–anti (χ^1, χ^2, −66°, −173°) and an anti–anti conformation (χ^1, χ^2, −179°, −168°, respectively) has been reported for another fully blocked Glu-Glu peptide, *N*-(benzyloxycarbonyl)-γ-ethyl-L-glutamyl-γ-ethyl-L-glutamic acid ethyl ester.[28] By comparison, the α form of glutamic acid[29] is anti–gauche (χ^1, χ^2, 178.2 (1)°, 68.3 (5)°, respectively) while the β form is gauche–gauche (χ^1, χ^2, −51.8(1)°, −73.1(2)°, respectively).[30] The ring strain of the cyclopropyl group is reflected in the widening of the exocyclic bond angles (all are greater than 116°) from tetrahedral values. This is particularly true for N15–C1–C2 (119.1 (3)°) and C1–C2–C5 (119.4 (3)°) due to the eclipsed conformation. The close proximity of N15 to C5 (2.960 (6) Å) causes a slight distortion from perpendicularity of the N15–C1–C4 plane from the plane of the cyclopropyl ring (dihedral angle 91.2 (3)°). The amide bond is trans (ω 171 (3)°). Molecules of **5** are H bonded in columns along the *a* axis through an N–H···O interaction [N15···O17 (1 + *x*, *y*, *z*) 2.879 (4); H15···O17 2.07 (4) Å]. The phenyl ring is twisted out of the plane of the amide group by 35.8 (2)° due to close contacts resulting from the H bonding.

Table III. Structures of Glutamic Acid Derivatives

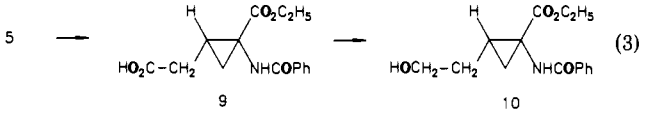


compd	R_1	R_2	R_3
11	CH_3	CH_3	COPh
12	CH_2CH_3	CH_2CH_3	COPh
13	H	CH_3	COPh
14	H	CH_2CH_3	COPh
15	CH_3	H	COPh
16	CH_2CH_3	H	COPh
t-Boc-L-Glu α-benzyl ester (**20**)	CH_2Ph	H	$COOC(CH_3)_3$

Cycloaddition of diazomethane to a dehydroamino acid followed by nitrogen extrusion has previously been observed to produce the cyclopropane with the same configuration as the starting olefin.[18,19] Our assignment of the *Z* configuration to olefin **3** and to cyclopropane **5** is therefore in accord with this experience.

Selective Protection. The vitamin K dependent carboxylase has been shown to recognize N-acylated glutamic acid α-esters as substrates.[17] We therefore sought to prepare such α-esters from our cyclopropane analogue of glutamic acid.

We first investigated the selective saponification of **5**. Treatment of the diethyl ester **5** with a stoichiometric amount of LiOH led to the formation of a single monoethyl ester accompanied by smaller amounts of diacid **6** and unreacted starting diester **5** (eq 3). This ester was unambigously identified as α-ethyl ester **9** by selective reduction of the acid to the primary alcohol using limiting diborane (B_2H_6). It is well-established that diborane reduces acids selectively in the presence of amides or esters.[31] Reduction of monoacid **9** with limiting diborane produced a single, monoalcoholic product, 2-hydroxyethyl-substituted-1-aminocyclopropane-1-carboxylate derivative **10**, identified through its characteristic ^1H NMR spectrum.



(3)

The exclusive hydrolysis of the γ-ester of **5** was unexpected, since the α-carboalkoxy group of an acylated amino acid is expected to be more reactive toward nucleophilic reagents than the γ-carboalkoxy due to the inductive effect of the acylamine. The hydrolysis of simple *N*-benzoylglutamic acid esters was therefore examined to determine the specificity for hydrolysis of the parent acylamino acid diester. Treatment of *N*-benzoylglutamic acid dimethyl ester (**11**) or *N*-benzoylglutamic acid diethyl ester (**12**) with 1 equiv of LiOH produces a major product identified as γ-ester, **13** or **14**, respectively (structures of the *N*-benzoylglutamic acids are presented in Table III). In each case the γ-ester was accompanied by a significant quantity

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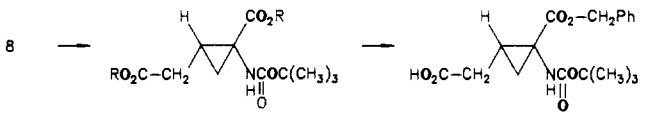
of *N*-benzoylglutamic acid. However, only a barely detectable (by TLC) quantity of the α-ethyl ester, either **15** or **16**, was produced. The products of the hydrolysis were identified chromatographically and through comparison to authentic materials. γ-Methyl ester **13** was synthesized from L-glutamic acid γ-methyl ester on treatment with benzoyl chloride.[32] *N*-Benzoyl-L-glutamic acid α-methyl ester (**15**) was produced by treating *N*-Benzoyl-L-glutamic acid with 1 equiv of diazomethane. *N*-Benzoyl-L-glutamic acid α-ethyl ester (**16**) was produced from methyl ester **15** by base-catalyzed transesterification. Finally, γ-ethyl ester **14** was isolated from the base-hydrolysis products and characterized. Thus, both members of each set of isomeric monoesters are known, were demonstrated to be chromatographically separable, and have been fully characterized. Therefore, the simple glutamic acid derivatives **11** and **12** were shown to predominately yield γ-esters **13** and **14** under conditions where the cyclopropane-containing compound **5** exclusively produces α-ester **9**. The difference in selectivity can be explained due to lowering of the reactivity of the α-ester in the cyclopropane-containing compounds by electron donation from the cyclopropane ring and due to increased steric congestion in the vicinity of the tetrasubstituted α-carbon.

Since *t*-Boc-L-glutamic acid α-benzyl ester was found to be the best of the simple glutamic acid derivatives as a carboxylase substrate,[17] we prepared the corresponding cyclopropyl glutamic acid derivatives (equation 4). Amino



17 R = H
18 R = $-CH_2Ph$
19

(4)

acid 8 was converted to its *tert*-butyloxycarbonyl derivative 17 on treatment with di-*tert*-butyl dicarbonate and further to dibenzyl ester 18 by reaction with excess phenyldiazomethane. The resulting Boc amino acid dibenzyl ester 18 was selectively saponified with a stoichiometric amount of LiOH to produce the desired Boc-protected methyleneglutamic acid α-benzyl ester **19**.

Enzymology. The synthesized α,β-cyclopropyl analogues of various glutamyl derivatives were utilized in a standard assay for vitamin K dependent carboxylase activity[33] to determine if they were substrates or inhibitors of the reaction. The *N*-benzoyl α-ethyl ester of α,β-methyleneglutamate (**9**) was found to be neither a substrate of the enzyme nor an inhibitor of the carboxylation of a commonly used substrate, *t*-Boc-Glu-Glu-Leu-OMe. The Glu analogue of this potential substrate, *N*-benzoyl-α-ethyl-L-glutamic acid (**16**), had low, but detectable, substrate activity which was not inhibited by a 100:1 ratio of the corresponding cyclopropyl analogue of glutamic acid. Preincubation of the enzyme for as long as 60 min with the *N*-benzoyl α-ethyl ester of α,β-methyleneglutamic acid (**9**) did not influence the subsequent carboxylation of *t*-Boc-Glu-Glu-Leu-OMe (data not shown). The *N*-benzoyl α-ethyl derivative was not a very effective substrate for the enzyme. The simple Glu derivative which has been demonstrated to have the greatest substrate activity[17] is *t*-Boc-Glu-Bz, and the cyclopropyl analogue of this derivative was shown to lack substrate activity or the ability to inhibit the carboxylation of the Glu derivative (Table IV).

Table IV. *N*-*t*-Boc-L-glutamic Acid α-Benzyl Ester and *N*-*t*-Boc-α,β-methyleneglutamic Acid α-Benzyl Ester as Substrates or Inhibitors of the Vitamin K Dependent Carboxylase[a]

Glu site substrate added	carboxylase activity, dpm
none	360 ± 10
2 mM *N*-*t*-Boc-α,β-methyleneglutamic acid α-benzyl ester (**19**)	300 ± 22
1 mM *N*-*t*-Boc-L-glutamic acid α-benzyl ester (**20**)	5800 ± 254
2 mM **19** + 1 mM **20**	5770 ± 187

[a] Incubations were at 17 °C for 30 min. Values are mean ± SD for four values.

Table V. Carboxylase and Epoxidase Activity with Glutamic Acid and α,β-Methyleneglutamic Acid Derivatives as Substrates[a]

substrate	carboxylase activity, dpm	epoxide formation, nmol
none	710 ± 22	4.3 ± 0.3
5 mM *N*-*t*-Boc-α,β-methylene-glutamic acid α-benzyl ester (**19**)	690 ± 12	5.1 ± 0.4
0.5 mM *N*-*t*-Boc-L-glutamic acid α-benzyl ester (**20**)	12600 ± 800	7.1 ± 0.4
5 mM **19** + 0.5 mM **20**	12200 ± 700	6.7 ± 0.8

[a] Incubations were at 17 °C for 60 min. Values are mean ± SD for four values.

The carboxylase catalyzes both the carboxylation of a glutamyl residue and the formation of vitamin K 2,3-epoxide,[34] and these two reactions are closely linked (eq 1). The data in Table V indicate that *t*-Boc-L-glutamic acid α-benzyl ester, but not its cyclopropyl analogue **19**, was capable of stimulating epoxide formation.

Discussion

The mechanism through which the vitamin K dependent carboxylase couples oxidation of a hydroquinone to carboxylation of glutamyl residues remains unknown. Despite searching, evidence that the hydroquinone functions as a CO_2 carrier has not been forthcoming.[35,36] A role for the coenzyme in the labilization of the γ-CH bond of the substrate glutamyl residue is supported by the observation that the reduced vitamin promotes an oxygen-dependent exchange of one of the γ-glutamyl hydrogens with solvent-water protons.[5-7] Proposals for activation of the glutamyl γ-CH include (1) deprotonation by base to produce a carbanion, (2) production of a γ-glutamyl free radical, (3) a hybrid mechanism in which a γ-glutamyl free radical is converted to a carbanion by its one-electron reduction, or (4) a concerted carboxylation.

Preliminary evidence that β-fluoroglutamyl-containing substrates undergo carboxylase-catalyzed elimination of HF, forming on olefin, supports the intermediacy of a carbanion on the γ-glutamyl carbon.[11] The formation of a carbanion by deprotonation of a carbon adjacent to a carboxylate or carboxylic acid (mechanism 1) would, however, require an extremely strong base. (The pK_a of the α-methyl proton of ethyl acetate is 25). This is a base strength far greater than that which could be anticipated for either an active site amino acid residue or hydroperoxide anion (pK_a values for model hydroperoxide anions range between 11.5 and 13[37]). A concerted carboxylation

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(mechanism 4) lowers the energy barrier to proton abstraction but still requires that an anionic transition state be formed adjacent to a carboxylate, an energetically unfavorable process. Carboxylation of a free radical intermediate (mechanism 2) explains the facile activation of the nonacidic glutamyl C–H but requires that CO_2 radicals be produced biochemically to complete the reaction via radical coupling. Although model reactions for such radical couplings have been noted,[9] the process is without precedent biochemically. A hybrid process (mechanism 3) recognizes that the existence of radical and carbanionic intermediates are not mutually exclusive. Such a hybrid process is attractive in that it offers a way to explain the removal of a nonacidic γ-glutamyl hydrogen (by free-radical abstraction) and at the same time explains the carboxylation as the result of the reaction of CO_2 with a carbanion.

The existence of a plausible mechanism in which both radical and anionic intermediates appear prompted us to attempt to use the intramolecular rearrangement of cyclopropyl analogues of glutamate to detect the radical. The α,β-methyleneglutamate analogue of Glu site carboxylase substrates did not incorporate $^{14}CO_2$ upon incubation with the enzyme, nor did they act as competitive inhibitors of the reaction. Preincubation of these analogues with the enzyme did not decrease activity, suggesting that they were not acting as mechanism-based irreversible inhibitors of the enzyme. The basic vitamin K dependent reaction is hydrogen abstraction, not carboxylation, and it is possible that the cyclopropyl analogues were acting as substrates but could not be detected because the mechanism was radical abstraction followed by rearrangement to yield a product that would not be carboxylated. The close association between epoxide formation and hydrogen abstraction[33] would suggest that if this were the case, the methyleneglutamyl derivative would stimulate epoxide formation. This was not observed.

The lack of substrate activity, inhibitory activity, or epoxidase stimulation would all suggest that substitutions at the α-carbon of the Glu site substrate prevent substrate binding. The enzyme is stereospecific[11] and carboxylates aspartyl residues poorly.[38] Although it is possible that an (*E*)-cyclopropylamino acid derivative would be recognized, it is more likely that a non-α-substituted probe capable of detecting a radical intermediate would be needed to test for this mechanism of carboxylase action.

Experimental Section

General Methods. Melting points were determined on a Thomas-Hoover apparatus and are uncorrected. Infrared absorption spectra were measured with a Perkin-Elmer 283B infrared spectrophotometer, with polystyrene as a standard. The proton NMR spectra were recorded at 90 MHz on a JEOL FX 90-Q spectrometer, at 300 MHz on a General Electric GN-300 spectrometer, or at 500 MHz on a General Electric GN-500 spectrometer. Carbon NMR spectra were determined at 22.5, 75, or 125 MHz, respectively. Chemical shifts are reported in ppm from an internal standard of TMS (for organic solutions) or sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4 (TSP) (for aqueous solutions) unless otherwise indicated. Assignment of ^1H and ^{13}C signals were accomplished with 2D-NMR techniques[25] and the attached-proton test.[39] Thin-layer chromatography was performed with precoated silica gel plates containing UV indicator (silica gel GHLF, 250-μm layer, purchased from Analtech, Newark, DE). The developed plates were examined under ultraviolet light, or the positions of bands were determined by exposure to I_2 vapors, by reaction with ninhydrin (1% ninhydrin in ethanol, followed by heating at 80 °C), or by charring after spraying the plate with a 50% solution of H_2SO_4 in H_2O. Preparative thin-layer chromatography utilized precoated silica gel plates with fluorescent indicator (2000-μm layer) purchased from Analtech (silica gel GF). Column chromatography was performed with silica gel (70–230 mesh) purchased from Aldrich Chemical Co. (Milwaukee, WI).

High-pressure liquid chromatography (HPLC) was performed with a system consisting of two LDC/Milton Roy constaMetric metering pumps, a LDC/Milton Roy Model 1601 gradient controller and dynamic mixer, and a Model 1203 A UV Monitor III fixed-wavelength detector operated at 254 nm. Preparative purification on HPLC utilized two Dynamax C-18 (Rainin Instruments, Woburn, MA) reversed-phase columns (10 mm × 250 mm each) and a guard column (10 mm × 50 mm) connected in series, with development with a mixture of acetonitrile and water (40:60) at a flow rate of 1.5 mL/min. Analytical HPLC was performed on a silica column (Whatman Partisil 10; 4.6 mm × 250 mm), developed with a mixture of hexane and 2-propanol (90:10) at a flow rate of 1.0 mL/min. The elemental analyses were provided by Galbraith Laboratories, Inc., Knoxville, TN.

Materials. α-Ketoglutaric acid, benzonitrile, *p*-toluenesulphonic acid monohydrate, and Diazald were purchased from Aldrich Chemical Co. (Milwaukee, WI). Benzene and tetrahydrofuran were dried by distillation from sodium/benzophenone. After distillation, the dry benzene was stored above 3A molecular sieves. Methylene chloride and chloroform were purified by distillation from P_2O_5 and stored above 3A molecular sieves. All other solvents were of reagent grade and used without purification unless otherwise specified.

Diethyl α-ketoglutarate (1) was prepared from α-ketoglutaric acid by using the method of Howarth and King:[40] R_f (silica gel, $CHCl_3$) 0.43; bp 92–94 °C (1.2 mmHg) [lit.[24] bp 165–168 °C (30 mmHg)].

Diethyl α,α-Dibenzamidoglutarate (2). Concentrated sulfuric acid (50 mL) was cooled to −10 °C and diethyl α-ketoglutarate (5.05 g, 0.05 mol) was added dropwise while stirring. When the addition was complete, benzonitrile (6.50 g, 0.05 mol) was added dropwise at such a rate that the temperature remained below −10 °C. When the addition was complete, the reaction mixture was stirred at −10 °C for 1 h and then at 0–5 °C for 6 h. By the end of this time, the reaction mixture had become very thick. The viscous mixture was poured slowly onto about 250 g of crushed ice with vigorous stirring. A white, gummy material separated, which was kept overnight at 0–5 °C whereupon the tacky material solidified to a white solid. This was filtered, washed with ice-cold water, dried, and recrystallized from ethanol to yield 7.5 g (70.4% theory) of white crystals: mp 129–130 °C; R_f (silica gel, $CHCl_3$) 0.15; IR (mull in mineral oil) 1740 ($COOC_2H_5$), 1660 ($NHCOC_6H_5$) cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.25 (t, J = 7 Hz, 3 H, CH_3), 1.33 (t, J = 7 Hz, 3 H, CH_3), 2.44 (distorted t, J = 9 Hz, 2 H, $CH_2CH_2COOC_2H_5$), 2.90 (distorted t, J = 7 Hz, $CCH_2COOC_2H_5$) 4.14 (q, J = 7 Hz, 2 H, OCH_2CH_3), 4.37 (q, J = 7 Hz, 2 H, OCH_2CH_3), 7.43–7.56 (m, 6 H, aromatic), 7.79–7.95 (m, 4 H, aromatic); ^{13}C NMR ($CDCl_3$) δ 13.92 (2 × OCH_2CH_3), 28.65 ($CCH_2CH_2COOC_2H_5$), 30.17 ($CCH_2CH_2COOC_2H_5$), 60.89 (OCH_2CH_3), 63.06 (OCH_2CH_3), 69.99 (quaternary), 127.03, 128.55, 132.07, 133.54 (aromatic), 166.97, 169.94, 172.49 (carbonyl). Anal. ($C_{23}H_{26}N_2O_6$) C, H, N.

Diethyl *N*-Benzoyl-α,β-dehydroglutamate (3). A 250-mL three-neck flask was fitted with a reflux condenser, a N_2 inlet, a vacuum inlet and charged with *p*-toluenesulphonic acid monohydrate (0.495 g, 0.0025 mol). The acid was dried by heating at 80–90 °C under vacuum for 12 h, at which time the flash was filled with nitrogen and maintained under a nitrogen blanket. Dry benzene (80 mL) was introduced via a syringe, followed by a solution of diethyl α,α-dibenzamidoglutarate (2; 8.52 g, 0.02 mol) in benzene (20 mL). The mixture was brought to reflux, and the progress of the reaction was monitored by TLC (silica gel, $CHCl_3$). When the starting material was consumed (usually 5 days), the reaction mixture was cooled in ice and filtered to remove benzamide. The filtrate was extracted twice with 50-mL portions of a 5% aqueous sodium bicarbonate solution and the organic phase was dried above anhydrous magnesium sulfate. After filtration and evaporation of solvent, the crude residue was purified by

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chromatography on a column of silica gel. Elution with a mixture of benzene and chloroform (75:25) resulted in the isolation of 4.52 g (74.3%) of a viscous oil (3), R_f (silica gel, $CHCl_3$) 0.18. A highly pure sample of 3 could be obtained with high-pressure liquid chromatography on a C-18 reversed-phase column developed with 2:3 acetonitrile–water at a flow rate of 1.5 mL/min. Product 3 emerged as a UV-absorbing peak after 35 min. Purity was confirmed with analytical high-pressure liquid chromatography (silica gel, hexane/2-propanol (90:10); t_R = 6.5 min).

Pure 3 was a colorless amorphous material: IR (mineral oil mull) 1730, 1660 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.26 (t, J = 7 Hz, 3 H, OCH_2CH_3), 1.33 (t, J = 7 Hz, 3 H, OCH_2CH_3), 3.35 (d, J = 9 Hz, 2 H, $CH_2C{=}C$), 4.19 (q, J = 7 Hz, 2 H, OCH_2CH_3), 4.29 (q, J = 7 Hz, 2 H, OCH_2CH_3), 6.94 (t, J = 9 Hz, 1 H $CH_2CH{=}C$), 7.50–7.58 (m, 3 H, aromatic), 7.80–8.04 (m, 2 H, aromatic); ^{13}C NMR ($CDCl_3$) δ 14.16 (OCH_2CH_3), 34.63 ($CH_2C{=}$), 60.85 (OCH_2CH_3), 61.83 (OCH_2CH_3), 126.73 ($CH_2C{=}C$), 127.38 (aromatic), 128.68 (aromatic), 131.98 (aromatic), 133.92 (aromatic), 141.78 ($C{=}C(NH)COO$), 164.16 (carbonyl), 165.19 (carbonyl), 170.72 (carbonyl); mass spectrum m/z 305 ($[M]^+$). Anal. ($C_{16}H_{19}NO_5$) C, H, N.

4-(Carbethoxymethyl)-3-benzamido-3-carbethoxy-4′-pyrazoline (4). A solution of diethyl N-benzoyl-α,β-dehydroglutamate (3; 0.92 g, 0.003 mol) in $CHCl_3$ (10 mL) was treated with an ethereal solution of diazomethane. The alcohol-free diazomethane, which was present in excess, was generated from 2.00 g of Diazald and was dissolved in 50 mL of ethyl ether. The resulting yellow solution was kept in a refrigerator (5 °C) overnight (18 h), treated with anhydrous $CaCl_2$ to destroy unreacted diazomethane, and filtered, and the solvent was evaporated. This material is generally used immediately in the next step. An analytical sample was obtained using preparative TLC; R_f (silica gel, $CHCl_3$) 0.17; IR (neat) 1740, 1760, 1560 (N=N) cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.16 (t, J = 7 Hz, 3 H, OCH_2CH_3), 1.33 (t, J = 7 Hz, 3 H, OCH_2CH_3), 2.46–2.55 (m, 2 H, CH_2CH_3), 2.99 (m, 1 H, CH_2CHCH_2), 4.10 (q, J = 9 Hz, 2 H, OCH_2CH_3), 4.40 (q, J = 7 Hz, 2 H, OCH_2CH_3), 5.01 (dd, J = 9 Hz, 2 H, $CH_2CHCH_2N{=}N$), 7.44–7.55 (m, 3 H, aromatic), 7.76–7.85 (m, 2 H, aromatic), 8.00 (s, 1 H, NH); ^{13}C NMR ($CDCl_3$) δ 14.16 (2 × OCH_2CH_3), 32.90 ($O_2CCH_2CHCH_2N{=}N$), 34.20 ($-O_2CCH_2CH-$) 60.86 (OCH_2CH_3), 63.67 (OCH_2CH_3), 84.58 ($CH_2CHCH_2N{=}N$), 101.70 (quat), 127.27 (aromatic), 128.79 (aromatic), 132.25 (aromatic), 133.80 (aromatic), 166.56 (carbonyl), 168.28 (carbonyl), 172.27 (carbonyl).

Ethyl 2-(Carbethoxymethyl)-1-benzamidocyclopropane-1-carboxylate (5). The entire quantity of pyrazoline 4 obtained starting from 3 mmol of protected dehydroglutamic acid 3 was dissolved in 75 mL of a mixture of diethyl ether and benzene (9:1) and placed in a 100-mL quartz tube (4 cm × 20 cm, fitted with a 24/40 joint), and the oxygen was purged by bubbling N_2 through the mixture for 15 min. A cold-finger condenser was inserted into the liquid from the top of the quartz tube, and ice–water was circulated to control the internal temperature as the solution was irradiated with ultraviolet light using a shortwave mercury irradiation apparatus. The progress of the reaction was monitored with TLC. When starting material was consumed, solvent was evaporated and the product was purified by column chromatography on silica gel (4 × 45 cm); product was eluted from the column with 1:1 benzene–$CHCl_3$, and 750 mg of 5 (78% yield, based on 0.003 mol of 3) was isolated. An analytical sample of 5 was obtained with preparative HPLC (C-18 reversed phase column, 2:3 acetonitrile–water, retention time 60 min) and recrystallized from CCl_4 and petroleum ether. Material so purified was homogeneous when examined by analytical HPLC (silica gel, hexane–2-propanol 9:1; t_R = 9.6 min): mp 94–96 °C; R_f (silica gel, $CHCl_3$) 0.09; IR (mineral oil) 1725, 1650 cm^{-1}; mass spectrum, m/z 319 ($[M]^+$); for ^1H and ^{13}C NMR, see Table I and II. Anal. ($C_{17}H_{21}NO_5$) C, H, N.

2-(Carboxymethyl)-1-benzamidocyclopropane-1-carboxylic Acid (6). Ethyl 2-(carbethoxymethyl)-1-benzamidocyclopropane-1-carboxylate (5; 160 mg, 0.50 mmol) was dissolved in methanol (1 mL), cooled in an ice bath, and treated with an ice-cold solution of LiOH (48 mg, 2 mmol) in water (1.5 mL). The resulting solution was stirred in a cold room (5 °C) for 24 h. The reaction mixture was extracted twice with $CHCl_3$ (5 mL), acidified to pH 2 with 1 N HCl, and saturated with ammonium sulfate, and the product was extracted into tetrahydrofuran (5 mL). The aqueous phase was extracted two more times with tetrahydrofuran (5 mL), and the combined organic extracts were dried above $MgSO_4$. The solvent was removed under reduced pressure and the residue was purified by preparative TLC (silica gel, 8:1:1 $CHCl_3$–CH_3OH–CH_3COOH). Product was crystallized from ethyl acetate–petroleum ether to yield 110 mg (83%) of a colorless solid: mp 215–217 °C dec; R_f (silica gel, 8:1:1 $CHCl_3$–CH_3OH–CH_3COOH) 0.61; IR (mineral oil mull) 1710, 1640 cm^{-1}; fast atom bombardment mass spectrum, m/z 264 ($[MH]^+$); ^1H NMR (5% CD_3OD in $CDCl_3$) δ 1.16 (dd, J = 6 and 7 Hz, 1 H), 1.97 (dd, J = 5 and 9 Hz, 1 H), 2.11 (m, 1 H), 2.33 (dd, J = 9 and 16 Hz, 1 H), 2.71 (dd, J = 5 and 16 Hz, 1 H), 7.4–7.6 (m, 3 H), 7.81–7.83 (m, 2 H); ^{13}C NMR δ 22.37, 24.37, 33.91, 38.08, 128.00, 129.63, 133.37, 133.75, 173.13, 176.55, 177.36.

Methyl 2-(Carbomethoxymethyl)-1-benzamidocyclopropane-1-carboxylate (7). 2-(Carboxymethyl)-1-benzamidocyclopropane-1-carboxylic acid (6, 65 mg, 0.25 mmole) was dissolved in ethyl alcohol (1 mL) and treated with a slight excess of diazomethane in alcoholic ether for 18 h at 5 °C. Excess diazomethane was destroyed by allowing the solution to stand above anhydrous $CaCl_2$ at room temperature for 2 h. The supernatant was removed, the solvent was evaporated under reduced pressure, and the product was purified with preparative TLC (silica gel, $CHCl_3$) to produce 60 mg (82% yield) of 7 as a viscous oil; R_f (silica gel, $CHCl_3$) 0.15; IR (neat) 1740, 1660 cm^{-1}; fast atom bombardment mass spectrum, m/z 292 ($[MH]^+$); for ^1H and ^{13}C NMR, see Tables I and II.

(Z)-2-(Carboxymethyl)-1-aminocyclopropane-1-carboxylic Acid (8). Ethyl 2-(carbethoxymethyl)-1-benzamidocyclopropane-1-carboxylate (5, 160 mg, 0.5 mmol) was suspended in 10 mL of 6 N HCl and heated in a water bath at 75 °C. The reaction was monitored by TLC (silica gel, 5:3:2 n-butanol–acetic acid–water) and stopped after 8 h, as soon as the ultraviolet-active amino acid derivatives were converted completely into a single ninhydrin-positive component. When hydrolysis was complete, the mixture was cooled in ice and extracted several times with ethyl acetate, and the aqueous solvent and acid were removed under reduced pressure. Product was redissolved in water and lyophilized to 88 mg (90%) of 8, as the hydrochloride. The product was purified by absorbing it onto a column (1 × 5 cm) of anion-exchange resin (^-OH form, Dowex 2-X8, 200–400 mesh, 1.2 mequiv/mL resin bed). The column was washed with water, and the product was eluted into 2 bed volumes of 1 N HCl. Solvent was evaporated under reduced pressure at room temperature. The product was next applied to a column (1 × 5 cm) of AG 50W-X8 cation-exchange resin (H^+ form, Bio-Rad Laboratories, Richmond, CA, 50–100 mesh, 1.7 mequiv/mL resin bed). The column was washed with water, and the product was subsequently eluted into 2 bed volumes of 2 N NH_4OH. Solvent was removed under reduced pressure at room temperature. Faint yellow impurities were removed by treatment of the aqueous solution with activated charcoal: R_f (silica gel, 5:3:2 n-butanol–acetic acid–water) 0.53; IR (mineral oil mull) 1640, 1615 cm^{-1}; fast atom bombardment mass spectrum, m/z 160 ($[MH]^+$); for ^1H and ^{13}C NMR, see Tables I and II. After this work had been completed, a synthesis of 8 was reported.[41] Neither spectroscopic nor physical properties for 8 were presented in this work.

Ethyl 2-(Carboxymethyl)-1-benzamidocyclopropane-1-carboxylate (9). Protected amino acid 5 (351 mg, 1.1 mmol) was dissolved in THF (2 mL) and water (1 mL), the solution was stirred magnetically in an ice bath, and cold aqueous LiOH was added (1 mL of a 1 M solution; 1 mmol). The resulting two-phase mixture formed a solution within 5 min. After stirring for 1 h at 0 °C, water was added and the mixture was extracted three times with ether (25 mL). Ether extracts were discarded. The aqueous phase was acidified with 1 N HCl (2 mL) and saturated with ammonium sulfate (30 g) and the product was extracted into THF (25 mL) followed by ether (25 mL). Aqueous phase was reacidified with 4 N HCl (4 mL) and reextracted twice with ether (25 mL). The combined organic extracts were dried above magnesium sulfate, filtered, and evaporated. The resulting oil was purified by column chromatography (silica gel, 60 g; 1%

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CH_3OH, 1% CH_3CO_2H in ethyl acetate), and a colorless oil (180 mg, 62% yield) was isolated which crystallized from benzene as fine white needles: mp 106.5–108.5 °C; R_f (silica gel, 1% CH_3OH, 1% CH_3CO_2H in ethyl acetate) 0.57; fast atom bombardment mass spectrum, m/z 292 ($[MH]^+$); ^1H NMR ($CDCl_3$) δ 1.18 (t, J = 7 Hz, 4 H, CH_2CH_3 and one cyclopropyl CH_2), 1.86 (m, 1 H, cyclopropyl CH_2), 2.1 (m, 1 H, cyclopropyl –CH–), 2.39 (dd, J = 9 and 16 Hz, 1 H, $-CH_2-$), 2.68 (dd, J = 6 and 16 Hz, 1 H, $-CH_2-$), 4.11 (q, J = 7 Hz, 2 H, CH_2CH_3), 7.3–7.4 (m, 3 H, aromatic), 7.81–7.86 (m, 2 H, aromatic).

Diborane Reduction of 9: Ethyl 2-(2-Hydroxyethyl)-1-benzamidocyclopropane-1-carboxylate (10). A solution of **9** (90 mg, 0.31 mmol) in dry THF (0.62 mL) was stirred magnetically in an ice bath under an N_2 atmosphere and treated with 0.31 mL of a 1 M solution of B_2H_6 in THF (0.31 mmol; Aldrich) added via a syringe. After 30 minutes, the reaction was quenched by adding ice-cold methanol dropwise until bubbling ceased, followed by 1% HCl in methanol (2 mL). Solvent was removed under reduced pressure, the acid addition and evaporation was repeated once, and finally two portions of THF (2 mL) were added and evaporated. The product was purified by column chromatography (silica gel, 60 g; 5% methanol in chloroform) and 50 mg (58.4% yield) of a colorless oil (**10**) was isolated: R_f (silica gel, 5% CH_3OH in $CHCl_3$) 0.46; mass spectrum, m/z 277 ($[M]^+$); ^1H NMR ($CDCl_3$) δ 1.00 (m, 1 H), 1.19 (t, J = 7 Hz, 3 H), 1.53 (m, 1 H), 1.73 (m, 1 H), 1.95 (dd, J = 9.3 and 5 Hz, 1 H), 2.07 (m, 1 H), 3.77 (m, 1 H), 3.87 (m, 1 H), 4.12 (q, J = 7 Hz, 2 H), 7.36–7.48 (m, 3 H), 7.86 (m, 2 H).

***N*-Benzoyl-L-glutamic Acid Dimethyl Ester (11).** *N*-Benzoyl-L-glutamic acid (2.51 g, 10 mmol; Sigma Chemical Co.) was added to a 1% (w/v) solution of HCl in methanol (50 mL; prepared by addition of 1 mL of acetyl chloride to 50 mL of methanol), and the solution was refluxed for 3 h. Solvent was evaporated under reduced pressure, the oil was dissolved in $CHCl_3$ (100 mL), and the organic solution was washed with 1 N $NaHCO_3$ (50 mL) and water (50 mL) and dried above anhydrous sodium sulfate. Removal of solvent left 2.57 g of product, crystallized from ethyl acetate and hexanes: mp 79–80 °C (lit.[42] mp 76–78.5 °C; lit.[43] mp 83 °C); ^1H NMR ($CDCl_3$) δ 2.17 (m, 1 H), 2.32 (m, 1 H), 2.48 (m, 2 H), 3.66 (s, 3 H), 3.79 (s, 3 H), 4.84 (m, 1 H), 7.47 (m, 3 H), 7.82 (m, 2 H).

***N*-Benzoyl-L-glutamic acid diethyl ester (12)** was prepared from *N*-benzoyl-L-glutamic acid and ethanolic HCl by using a procedure identical with that used for the dimethyl ester. Product was crystallized from ethyl ether and hexanes; mp 70–72 °C (lit.[42] mp 73–74 °C); ^1H NMR ($CDCl_3$) δ 1.22 (t, J = 7 Hz, 3 H), 1.31 (t, J = 7 Hz, 3 H), 2.16 (m, 1 H), 2.33 (m, 1 H), 2.47 (m, 2 H), 4.12 (q, J = 7 Hz, 2 H), 4.25 (q, J = 7 Hz, 2 H), 4.81 (m, 1 H) 7.45 (m, 3 H), 7.81 (m, 2 H).

***N*-Benzoyl-L-glutamic Acid γ-Methyl Ester (13).** L-Glutamic acid γ-methyl ester (4.8 g, 0.03 mol; Aldrich) was suspended in dry THF (40 mL) and treated with benzoyl chloride (5.0 g, 0.036 mol) at reflux for 10 h. The reaction was cooled, chloroform was added (20 mL), and the mixture was kept in a refrigerator for 3 h. The mixture was filtered to remove the precipitated starting material (2.4 g, 50% recovery). Solvent was removed from the filtrate under reduced pressure, and the residue was dried in vacuo overnight to yield a semisolid, which was recrystallized twice from chloroform/ether to yield 3.7 g (46%) of *N*-benzoylglutamic acid γ-methyl ester: mp 132–133 °C (lit.[32] mp 107 °C); IR (mineral oil mull) 3450–3480 (HO), 3290 (NH), 1730–1750 (COOR), 1640 (–CONH–) cm^{-1}. Anal. ($C_{13}H_{15}NO_5$) C, H, N.

Selective Saponification of *N*-Benzoyl-L-glutamic Acid Dimethyl Ester: *N*-Benzoyl-L-glutamic Acid γ-Methyl Ester (13). The hydrolysis of *N*-benzoyl-L-glutamic acid dimethyl ester (307 mg, 1.1 mol) was conducted with limiting LiOH and followed a procedure identical with that used in the production of **9**. Analysis by TLC and column chromatography used silica gel and 1% CH_3OH, 1% CH_3CO_2H in ethyl acetate. A single major product isographic with authentic γ-methyl ester (R_f 0.39) was accompanied by a minor product corresponding to *N*-benzoyl-L-glutamic acid (R_f 0.15) and traces of a material isographic with the α-methyl ester (R_f 0.6). The major product was isolated by column chromatography (90 mg; 34% yield) and its identity was established by comparison with authentic **13** produced by benzoylation of glutamic acid γ-methyl ester.

Selectlve Saponification of *N*-Benzoyl-L-glutamic Acid Diethyl Ester: *N*-Benzoyl-L-glutamic Acid γ-Ethyl Ester (14). The hydrolysis of *N*-benzoyl-L-glutamic acid diethyl ester (337 mg, 1.1 mmol), the TLC analysis of the products, and the chromatographic isolation of the major product followed procedures identical with those used in the production of **9**. The major product **14** (R_f 0.44) was accompanied by *N*-benzoyl-L-glutamic acid (R_f 0.15) and a faint spot isographic with *N*-benzoyl-L-glutamic acid α-ethyl ester (R_f 0.61). The major product **14** was isolated (100 mg; 36% yield) as an oil (for racemate, lit.[44] mp 114–115.5 °C) and identified spectroscopically as the monoethyl ester; ^1H NMR ($CDCl_3$) δ 1.05 (t, J = 7.1 Hz, 3 H, OCH_2CH_3), 1.96 (m, 1 H, $-CH_2CH_2CH-$), 2.23 (m, 3 H), 3.88 (q, J = 7.1 Hz, 2 H, OCH_2CH_3), 4.33 (m, 1 H, CH_2CH_2CH), 7.2–7.35 (m, 3 H, COPh), 7.76–7.79 (m, 2 H, COPh); fast atom bombardment mass spectrum, m/z 280 ($[MH]^+$).

***N*-Benzoyl-L-glutamic Acid α-Methyl Ester (15).** A solution of *N*-benzoyl-L-glutamic acid (1.255 g, 5 mmol; Sigma Chemical Co.) in THF (25 mL) was stirred, cooled in an ice bath, and treated with 21 mL of a solution of diazomethane (0.21 M, prepared as an ethereal solution from Diazald; 4.4 mmol), added dropwise over about 10 min. The resulting colorless solution was allowed to stand for 10 min, at which time the solvent was removed in vacuo. The single major product was purified by column chromatography (silica gel; 1% CH_3OH, 1% CH_3CO_2H in ethyl acetate) to produce 0.64 g of a colorless oil, which crystallized from benzene: mp 79–81 °C; ^1H NMR ($CDCl_3$) δ 2.15 (m, 1 H), 2.33 (m, 1 H), 2.52 (m, 2 H), 3.79 (s, 3 H), 4.85 (m, 1 H), 7.4–7.52 (m, 3 H), 7.8 (m, 2 H). Anal. ($C_{13}H_{15}NO_5$) C, H, N.

***N*-Benzoyl-L-glutamic Acid α-Ethyl Ester (16).** A solution of **15** (265 mg, 1 mmol) in ethanol (5 mL) and 1.2 equiv of sodium ethoxide (1.2 mL of 1 M sodium ethoxide) was stirred at ambient temperature for 1.5 h and then poured into water (20 mL) and extracted once with ether (25 mL). The aqueous layer was acidified with 4 N HCl and product was extracted into ethyl acetate (3 portions of 25 mL each), dried above magnesium sulfate, filtered, and evaporated. The resulting oil was purified by column chromatography (silica gel, 20 g; 1% CH_3OH, 1% CH_3CO_2H in ethyl acetate), and the single UV-active product was isolated to produce 280 mg (100% yield) of a colorless oil [for racemate, gum, lit.[45] bp 150–160 °C, (0.01 mmHg)]: ^1H NMR ($CDCl_3$) δ 1.18 (t, J = 7.2 Hz, 3 H, OCH_2CH_3), 2.02 (m, 1 H, CH_2CH_2CH), 2.18 (m, 1 H, CH_2CH_2CH), 2.38 (m, 2 H, CH_2CH_2CH), 4.11 (q, J = 7.2 Hz, 2 H, OCH_2CH_3), 4.72 (m, 1 H, CH_2CH_2CH), 7.29 (m, 3 H, COPh), 7.7 (m, 2 H, COPh).

2-(Carboxymethyl)-1-[(*tert*-butyloxycarbonyl)amino]-cyclopropane-1-carboxylic Acid (17). A mixture of **8** (140 mg, 0.89 mmol) in 1 N NaOH (2 mL) and *tert*-butyl alcohol (1 mL) was stirred vigorously as di-*tert*-butyl dicarbonate (0.262 g, 1.2 equiv) was added dropwise via a pipette and then rinsed with *tert*-butyl alcohol (1 mL). After stirring for 3 days at ambient temperature, the mixture failed to form a solution and analysis by TLC (silica gel, *n*-butanol–CH_3CO_2H–H_2O, 5:3:2, I_2) indicated unreacted starting material (R_f 0.51) and a single new component (R_f 0.85). The solvent was removed in vacuo; the residue was dissolved in water (15 mL) and extracted three times with ethyl acetate (10 mL). The aqueous phase was acidified with $NaHSO_4$, and the product was extracted into ethyl acetate (3 × 10 mL). Organic extracts were dried briefly above sodium sulfate, filtered, and evaporated, resulting in isolation of **15** as a colorless oil (100 mg: 44% yield). The ^1H NMR in $CDCl_3$ showed the expected high-field singlet for the *t*-Boc group at 1.45 ppm and the characteristic cyclopropyl resonances. This material was converted directly to dibenzyl ester **16** without further purification or characterization.

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Benzyl 2-[(Carbobenzyloxy)methyl]-1-[(*tert*-butyloxycarbonyl)amino]cyclopropane-1-carboxylate (18). A solution of 17 (250 mg, 0.97 mmol) in ethyl acetate (10 mL) was stirred magnetically at ambient temperature and treated with a solution of phenyldiazomethane (2 mmol) in ether. The red color of the diazoalkane was bleached within 1 h, at which time a second portion of phenyldiazomethane (0.2 mmol) was added. The reaction was allowed to continue stirring for 1 h, at which time a pink color persisted. Solvent was evaporated in vacuo and the resulting oil was purified by column chromatography (silica gel, 20 g; hexane and ethyl acetate, 4:1). The dibenzyl ester (180 mg, 42% yield) was isolated as an oil which crystallized on standing: R_f (silica gel; hexane and ethyl acetate, 4:1) 0.17; fast atom bombardment mass spectrum, m/z 440 ($[MH]^+$); ^1H NMR ($CDCl_3$), δ 1.06 (m, 1 H, cyclopropyl CH_2), 1.38 (s, 9 H, $C(CH_3)_3$), 1.82 (m, 1 H, cyclopropyl CH_2), 2.04 (m, 1 H cyclopropyl CH), 2.56 (m, 2 H, exocyclic CH_2), 5.14 (m, 4 H, OCH_2Ph), 7.32–7.33 (2 s, 10 H, OCH_2Ph). Anal. ($C_{25}H_{29}NO_6 \cdot {}^1/_4H_2O$) C, H, N.

Benzyl 2-(Carboxymethyl)-1-[(*tert*-butyloxycarbonyl)amino]cyclopropane-1-carboxylate (19). A solution of 18 (180 mg, 0.41 mmol) in THF (2 mL) and water (0.5 mL) was stirred in an ice bath as 0.3 mL of ice-cold 1 M LiOH (0.36 mmol) was added. Subsequently, 3 mL of water and 1.5 mL of THF was added to the two-phase mixture, but this failed to produce a solution. Finally, after 2 h, more THF was added (2 mL) and a solution resulted. The reaction was allowed to stir overnight at 4 °C. The next day, 25 mL of water was added and the resulting mixture was washed three times with ether (25 mL). The washes were discarded. The aqueous phase was acidified with 1 M HCl (2 mL) and saturated with ammonium sulfate (30 g) and the product was extracted into THF (25 mL) and then ether (25 mL). The aqueous layer was reacidified with 4 N HCl (4 mL) and reextracted two times with ether (25 mL). The combined organic extracts were dried above magnesium sulfate, the solvent was removed, and the product was isolated by column chromatography (silica gel, 20 g; 1% CH_3OH, 1% CH_3CO_2H in ethyl acetate), producing 50 mg (40% yield) of 17 as a colorless oil; R_f (silica gel, 1% CH_3OH, 1% CH_3CO_2H in ethyl acetate) 0.65; fast atom bombardment mass spectrum, m/z 350 ($[MH]^+$); ^1H NMR ($CDCl_3$) δ 1.06 (m, 1 H, cyclopropyl CH_2), 1.41 (s, 9 H, $C(CH_3)_3$), 1.81 (m, 1 H, cyclopropyl CH_2), 2.03 (m, 1 H, cyclopropyl CH), 2.47 (m, 1 H, exocyclic CH_2), 2.64 (m, 1 H, exocyclic CH_2), 5.13 (m, 2 H, OCH_2Ph), 7.32 (s, 5 H, Ph).

Vitamin K dependent carboxylase activity was assayed in liver microsomes prepared from vitamin K deficient rats essentially as described by Wood and Suttie.[33] When vitamin K epoxide formation was measured, microsomes were prepared from vitamin K deficient rats given 1 mg of phylloquinone (ic) 15 min before they were killed. To measure epoxide formation, 0.5 mL of reaction mixture was quenched by 2 volumes of 3:2 2-propanol–hexane (v/v), mixed with a vortex stirrer for 30 s, cooled to 4 °C for 15 min, and mixed again for 30 s. The samples were centrifuged at low speed, and the hexene layer was removed and dried under N_2. The residue was redissolved in 0.2 mL of methanol and injected on a Zorbax C-18 HPLC column and the column was developed with 100% MeOH. Peak areas were converted to nanomoles by comparison to a standard curve generated at the same time.

X-ray Experimental for 5. Crystals grew in long colorless rods by slow evaporation from a CCl_4–hexane solution. The data crystal was cut from a larger crystal and had dimensions 0.19 × 0.39 × 0.56 mm. Data were collected at room temperature on a Nicolet P3 diffractometer, with a graphite monochromator and Mo Kα radiation (λ = 0.71073 Å). Crystal system is orthorhombic and the space group is $P2_12_12_1$ as uniquely determined from systematically absent reflections. This is a chiral space group, and therefore, **5** is spontaneously resolved upon crystallization. The lattice parameters were obtained from least-squares refinement of 50 reflections with $20.5 < 2\theta < 26.1°$; a = 5.0335 (4) Å, b = 16.059 (1) Å, c = 21.414 (1) Å, V = 1731.0 (2) Å^3, ρ = 1.22 g cm^{-3} for Z = 4, $F(000)$ = 680. Data were collected with the ω scan technique (4731 reflections, 2354 unique, R_{int} = 0.0161 from averaging hkl and h, $-k$, $-l$ reflections), 2θ range 4.0°–55.0°, 1° ω scan at 4°–8°/min. (h = −3 → 6, k = −20 → 20, l = −27 → 27). Four reflections (0,0,10; 1,3,2; −1,−1,−5; 0,−6,0) were remeasured every 96 reflections to monitor instrument and crystal stability. Maximum decay correction <1%. Data were corrected for Lp effects but not for absorption (μ = 0.8440 cm^{-1}). Reflections having $F_o < 4\sigma(F_o)$ were considered unobserved (824 reflections). The structure was solved by direct methods (SHELXTL-PLUS, Nicolet XRD, 1987)[46] and refined by full-matrix least-squares procedures (Sheldrick, 1976)[47] with anisotropic thermal parameters for the non-H atoms. The ethyl group atoms exhibit large thermal motion and the resulting C–C bond lengths are quite short (C9–C10, 1.446 (11) Å; C13–C14, 1.396 (10) Å; normal Csp^3–Csp^3 bond length is 1.54 Å). Ethyl group H atoms were calculated and refined with thermal parameters riding at 1.2 × U_{eq} of the relevant C. All other H atoms were obtained from a ΔF map and refined with isotropic thermal parameters. A total of 252 parameters were refined. $\sum W(|F_o| - |F_c|)^2$ minimized, where $w = 1/(\sigma(F_o))^2$ and $\sigma(F_o) = 0.5kI^{-1/2}[(\sigma(I))^2 + (.02I)^2]^{1/2}$. Intensity, I, is given by ($I_{peak} - I_{background}$) × (scan rate); 0.02 is a factor to downweight intense reflections and to account for instrument instability; k is the correction due to Lp effects and decay. $\sigma(I)$ is estimated from counting statistics: $\sigma(I) = [(I_{peak} + I_{background})^{1/2} \times$ (scan rate)]. Final R = 0.0618 for 2354 reflections, wR = 0.0580 (R for all reflections = 0.0973, wR for all reflections = 0.0644) and a goodness of fit = 1.706. Maximum $|\Delta/\sigma| < 0.1$ in the final refinement cycle, and the minimum and maximum peaks in the final ΔF map was −0.20 and 0.23 e/Å^3, respectively. The structure analogous to the L-amino acid is shown in Figure 1 although discrimination between enantiomorphs could not be made on the basis of X-ray results. Neutral atom scattering factors were used for all atoms,[48,49] with anomalous-dispersion corrections for the non-H atoms.[50] Values for the linear absorption coefficient were taken from the International Tables for X-ray Crystallography.[51] The least-squares planes program was supplied by Cordes;[52] other computer programs are listed elsewhere.[53]

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Supplementary Material Available: Tables describing the unit-cell packing, fractional coordinates and isotropic thermal parameters, anisotropic thermal parameters, bond length and angles, torsion angles (11 pages); observed and calculated structure factor amplitudes for **5** (15 pages). Ordering information is given on any current masthead page.

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